IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Bevilaqua et al.	Atty Docket:	2331/112
Serial No.:	09/821,850	Art Unit:	1631
Date Filed:	March 29, 2001	Examiner:	Allen, M. P.

Invention: SYSTEMS AND METHODS FOR EVALUATING A BIOLOGICAL CONDITION OR AGENT USING SELECTED GENE EXPRESSION PROFILES

#21

Commissioner for Patents
Washington, DC 20231

DECLARATION OF VICTOR V. TRYON, Ph.D. IN SUPPORT OF APPLICANTS' RESPONSE
[37 C.F.R. § 1.132]

Dear Sir:

In response to the Office Action mailed December 24, 2002, in the above-reference matter, I hereby declare as follows:

1. My name is Victor V. Tryon, Ph.D. I am Vice-president of Technology and Bioinformatics, of Source Precision Medicine, Inc., the assignee herein, and one of the inventors of the subject matter of the above patent application. I am an inventor or co-inventor of a substantial number of patents involving (among other things) gene expression profiling for diagnostic and therapeutic goals, and related materials and methods. My further credentials are set forth in my Curriculum Vitae, which is attached as Exhibit A hereto.

2. I have read the action of December 24, 2002. This declaration is provided to clarify the record that someone of ordinary skill in the art would be able to practice the claimed invention based on the application as written (enablement) and also to set forth the

distinguishing differences between the technology and methods disclosed in the Rodriguez-Antona reference and those disclosed and claimed in the present application.

THE INVENTION IS ENABLED BY THE SPECIFICATION

General Information

3. The Central Dogma of Molecular Biology is an often-cited concept that is actually more of a model or teaching paradigm than actual fact. The Central Dogma holds that the heritable information flow in biology is from DNA to RNA and then to protein. That is, DNA is the heritable material and is transcribed into RNA as needed in development or life. RNA serves as a temporal and spatial shuttle of this information from the nucleus to cytoplasm where inherited information is transformed from nucleic acid into enzymes, structural building blocks and other types of protein. This concept dates from 1953 and the elucidation of the helical, anti-parallel, self-pairing structure of DNA by Watson, Crick and Franklin, but is founded on even more fundamental experiments conducted in the 1920's and 1930's.

4. Even the invention of polymerase chain reaction (PCR) by Kary Mullis in 1984/85 was founded on the self-replicating nature of the DNA molecule deduced in 1953 from the physical and chemical structure of helical, double-stranded DNA. Between the Watson and Crick reported discovery in 1953 and Mullis's invention of PCR in 1984, DNA was often "replicated" experimentally by the addition of a short, polynucleotide polymer called a primer, an enzyme (DNA polymerase), and the four foundational nucleotides, ATP, CTP, GTP, TTP. The result of this experimental replication was single-stranded DNA complementary to the starting strands of the starting material, the *double-stranded DNA template.*

5. Mullis' remarkable invention was the addition of a second primer complementary to the second strand of the double-stranded DNA and both distal and anti-parallel to the first primer. With this single addition, all the biological conditions necessary to permit the self-replication of DNA were met and the result was multiple (many to billions; the amount dependent on the thermodynamic conditions), double-stranded copies of the original or template DNA. Because the result of this procedure (PCR) is few to billions of additional molecules of double-stranded DNA, identical (within natural error) to the starting DNA material or template, this process has been called DNA amplification. The resulting number of identical copies depends on the number of "cycles" of amplification that are conducted. One cycle consists of primers annealing to complementary strands, new DNA synthesis by DNA polymerase and disruption of the stable strands through denaturation.

6. PCR then requires a double-stranded DNA molecule as a starting template. Single-stranded RNA is not suitable starting material for PCR (i.e., DNA amplification). Amplification by PCR results in many, many similar double-stranded DNA molecules. The methods described in this application are based on quantifying single-stranded RNA molecules as reflection of an underlying biological condition. How then is PCR used to quantify these single-stranded RNA molecules?

7. For some time (decades) scientists have known that RNA also may be a form of heritable nucleic acid. Examples in today's common news include primarily viruses such as HIV, the virus responsible for AIDS, and corona virus, the virus responsible for SARS or Sudden Acute Respiratory Syndrome. Here, contrary to the Central Dogma, the viral RNA encodes the heritable instructions. DNA does not typically exist in these viruses.

8. The viruses similar to HIV require an intermediate step of reverse transcription of the RNA into a double-stranded DNA or DNA-like molecule. This reverse transcription (RT; single stranded RNA to double-stranded DNA or DNA:RNA hybrid) is accomplished using a RNA-dependent, DNA polymerase more simply known as reverse transcriptase. The enzyme is either carried with the virus or encoded for replication. This conversion of single-stranded RNA to double stranded DNA has been known and used experimentally for decades.

9. Experimentally, the "reverse" transcription of RNA to DNA is typically accomplished in a single, non-cyclic, isothermic reaction, but is robust enough to be performed simultaneously with the DNA amplifications reactions. However performed, the result is transformation of a single-stranded RNA, such as mRNA (message), a substrate not suitable for amplification by PCR, into a double-stranded DNA molecule now suitable for enzymatic amplification by PCR

10. Reverse transcriptase is a reproducible activity dependent primarily on substrate availability, temperature, and activity or number of enzyme molecules. The experimental procedures described in the reference cited by the examiner (Rodriquez-Antona et al, 2000) deal with the replicability of this step (reverse transcription; that is, RNA to DNA). In my scientific view, reproducibility and other parameters contributing to variability of this step were well established in the art by the time of this application, as evidenced by hundreds of other publications over decades and were not the subject of the invention described in the application.

11. The settled nature of the efficiency of reverse transcriptase activity makes the examiner's citation, and others similar to that cited work, irrelevant to the discussion of

amplification efficiency in this application. That is, reproducibility or efficiency of the reverse transcriptase step is (1) not the same as amplification efficiency (as used in the application or the established art) and (2) was well established in the art prior to submission of this application for uses similar to those in this application and (3) is misapplied as germane to the question of anticipation of the disclosed invention.

Design of Primer/Probe Sets

12. Perhaps the most authoritative collection of methods concerning the quantitative aspects of analysis and underlying design methods of PCR and RT-PCR known to me in 1999, and through the present day, is found in the molecular medicine series entitled *Quantitative RT-PCR*; Paul D. Siebert, Chapter 4, pp. 61-86; *In*: Methods in Molecular Medicine, Volume 26; Quantitative PCR Protocols; B. Kochanowski and U. Reischl (Eds.) Humana Press; Totowa, New Jersey; 1999; ISBN 0-89603-5218-2 On p. 62 of that reference, Siebert identifies the known (as of 1998-1999) factors that affect the efficiency of amplification (*sensu stricto*, i.e., as known in the art, not as applied by the examiner).

13. The known factors affecting amplification efficiency included:

1. The sequence being amplified
2. The sequence of the primers
3. The length of the sequence being amplified
4. Impurities in the sample

The inventor's believe it is important to note, that *each* of the known factors affecting amplification efficiency identified by Professor Siebert is addressed specifically in the application.

(1) **The sequence to be amplified:**

"The primer probe should not amplify genomic DNA or transcripts or cDNA from related but biologically irrelevant loci." (Specification, p. 26, line 30)

(2) **The sequence of the primers:**

"The reverse primer should be complementary to the coding DNA strand; located across an intron-exon junction, with not more than three bases of the three-prime end of the reverse primer complementary to the proximal exon." (Specification, p. 26, line 25) See also the reference material cited in the application, and additional information within, for example, page 181 to 182; *Primer Selection*; McCreedy, B. J.; Detection of Viral Pathogens Using PCR Amplification; In, Molecular Methods for Virus Detection; D.L. Wiebrauk and D.H. Farkas (Eds); 1995; Academic Press; San Diego, CA.

(3) **The length of the sequence being amplified:**

"The primer probe should amplify cDNA of less than 110 bases in length." (Specification: p. 26, line 29)

(4) **Impurities in the sample:**

design programs in common use today and in the past rarely correlate well with actual results.

16. The Examiner's assertion that "one of ordinary skill in the art would be *unable to reproduce* any of the experiments presented in the figures as insufficient information in the specification is provided do so" *is not true* in the Applicants' experience. See Detailed Action; page 6, lines 3-5, emphasis added. On some occasions in the Applicants' laboratory separate individuals, designing primer-probe sets for the same locus, have independently arrived at the same primer-probe combination using the rules identified in the specification and common skill.

17. *A priori* there is no necessity for a single, sequence-specific solution to the primer-probe requirement. In some cases there may only be a single, unique solution. But again, depending on the length of target sequence and the exon complexity, there may exist multiple combinations that meet the requirements of the invention.

18. Despite the "class" rather than sequence-specific solution to the primer-probe requirement, there exists only a very small number of primer-probe combinations that fulfill the requirements of the invention. The smallest targets may only have one solution The longest targets may have perhaps 10 or fewer.

19. In addition to the factors specified by Siebert et al., we identified at least one other controllable factor affecting amplification efficiency. Primer template ratios (see application, page 26, line 7) were found by us to affect the routinely achievable levels of amplification efficiency. We determined that to achieve highly reproducible amplification efficiencies, it is necessary to maintain a known and reproducible ratio for the single,

unchangeable reaction conditions subjected to all of our amplification reactions in a selected panel.

20. In our experience, the acceptable ratio varies over many fold depending on reaction temperatures, salt concentrations and other parameters. However, as disclosed in the application, we found the selected panel to be uniquely informative if and only if a single set of such reaction conditions were maintained for all assayed loci (see application, page 24, lines 1-4, generally). That is, a single set of reaction conditions is applied to all constituents of the selected panel resulting in a calibrated profile of gene expression.

Evaluation of a Biological Condition

21. Contrary to the Examiner's assertion that the methods claimed in the present application are not enabled for evaluating a biological condition, once a profile data set has been obtained, it is necessarily the case that a biological condition has been evaluated. Taking someone's temperature allows evaluation of the presence or absence of a fever based on the number measured by the thermometer.

22. In an analogous manner, taking someone's blood pressure, or heart rate, or blood glucose level, or performing countless other diagnostic measurements, allows one skilled in medicine to evaluate a biological condition. Any of the above measurements, obtained by someone lacking in medical training is simply a number. To those skilled in the art of medicine, however, a deviation in someone's blood pressure level, glucose level, or heart rate, for example, facilitates evaluation of a biological condition, whether it be anxiety, arterial bleeding, arteriosclerosis, diabetes, or cardiacarrhythmia.

23. Although taking someone's temperature, or blood pressure, or blood glucose level or heart rate is a single measurement, the presently claimed invention is analogous in that a matrix of measurements is used to evaluate a biological condition, rather than a single measurement (providing more data, in a manner like using blood pressure, temperature, and heart rate in combination).

Amplification of Proteins

24. The concepts of precision, specificity and calibration of mRNA are not limited to just mRNA. As defined in the application, protein, like RNA, is a gene expression product. That is, the gene codes for a unique peptide sequence. Even though, like RNA, the protein to gene relationship can be described as many to one, nonetheless there is only a defined number of possible RNA or peptide products of a gene.

25. There are a number of ways to experimentally and diagnostically define a biological condition. For some biological conditions, the gene sequence may be sufficient, for example, with some forms of sickle cell anemia or cystic fibrosis. In other conditions, a measurable change in mRNA level of several constituents of a selected panel of constituents may be sufficient. In still other conditions the number of expressed proteins, both type and quantity, may be important. Even though gene sequence, mRNA levels, and expressed protein are relatable to the same gene in some definable way, the biological questions are many and the number of localized compartments in the human host is nearly infinite, e.g., cell vs. tissue, blood vs. cerebral spinal fluid, etc.

26. In the application we have stated that the Applicants' way to define the biological condition is to know with high precision and in a calibrated fashion (gene product to gene product) the relationship of a selected number of expression products.

27. State-of-the-art methods for quantifying expression products, whether RNA or protein, are remarkably similar at the physical (quantum) level. It may seem those unfamiliar in the art that since RNA is made up of nucleic acids and proteins are comprised of amino acids that the methods for detection, analysis and quantitation would necessarily be different. On closer examination it is obvious that both entities are polymeric (i.e., built from repeated chemical building blocks) acids; organic (i.e., carbon-based) compounds with positive charges and soluble in physiologic solutions.

28. In the inventors' view, those unfamiliar with the methods of analyzing and quantitating DNA and protein, would be more amazed at the commonality of methods rather than how the methods are uniquely different. This commonality has been appreciated for decades by those trained in physical biochemistry (see, or example, Friefeilder, Physical Biochemistry, 1984) but has been reinforced more recently by the publication of methods for the induced replication of prions (infectious proteins) that the authors and reviewers have described as analogous to gene amplification by PCR (see, for example, Saborio GP, Permanne B and C. Soto; Nature; 411:810-813; 2001; Soto, C, GP Saborio, and L. Andrews, Trends in Neurosciences; 25:390-394; 2002 and Telling, G.; *Protein-based PCR for prion diseases*; Nature Medicine; 7:778-779; 2001, attached as Exhibits C, D, and E, respectively).

29. In the application we have emphasized the need for precision and gene-to-gene calibration to accurately describe the biological condition using RT-PCR. In the application, see page 27, lines 13-16, we observed that if similar strategies were applied to commonly used measures of proteins such as ELISA, these strategies would result in the accurate description of the biological condition based on selected panels of proteins.

30. The fundamental concept of an ELISA is that there is a definable ratio between the number of analytes (protein molecules) and the number of antibodies bound. In the practical world, the *absolute* number of bound antibodies is impossible to determine. So in practice a detectable (countable) reporter molecule (or enzyme) is added to the antibody. There is now a definable ratio between the number of proteins and the number of detectable (quantitatable) reporter events. The reporter molecule amplifies the signal. The signal is equal to the number of antibodies bound to the cognate protein.

31. The fundamental concept of quantitative PCR is that there is a definable ratio between the number of double-stranded targets (i.e., copies of the mRNA produced by reverse transcription) and the number of reporter molecules bound or incorporated. In the practical world, it is impossible to determine the number of signal molecules (the probe) bound (or incorporated into) to double-stranded targets. The reporter molecule (the probe) amplifies the signal. The signal is equal to the number of probe molecules bound (incorporated) to the cDNA target (where cDNA is the DNA resulting from conversion of the single-stranded RNA to double-stranded DNA).

32. In the practical world, neither the antibody signal in the ELISA nor the probe signal in quantitative PCR is detectable without additional amplification. Signal is amplified in the ELISA by adding additional reporter-bearing antibodies (i.e., more antibodies, more signal generated). Signal is amplified in quantitative PCR by replicating the number of double-stranded products that incorporate signal (i.e., the more products, the more signal).

33. If the efficiency of the amplification step in either ELISA or PCR is not controlled, then each constituent of a selected panel would have a different ratio between

starting targets (the biological condition) and the reporter. This results in a lack of calibration between gene product "A" and gene product "B". This is the state-of-the-art today for antibody-based protein methods including ELISAs, as well as quantitative PCR.

34. The claimed invention in the present application shows how to produce gene-product-to-gene-product calibration by providing guidance in how to control the efficiency of the signal amplification process, and those skilled in the art would understand how this works in the context of protein expression or mRNA production. Since the invention of ELISA and PCR, guidance for increasing specificity has been provided in thousands of journal articles and hundreds of protocol manuals. That is, understanding control over specificity is a minimal requirement to be perceived as skilled in the art.

Undue Experimentation

35. It has historically been, and continues to be, standard operating procedure in laboratories practicing PCR today that determination of amplification efficiency is experimentally derived or confirmed. That is, the invention requires some minimal experimentation. Minimal in this context is in terms of time, complexity of method, type of analysis or cost.

36. Common practice for every skilled laboratory applying RT-PCR to human specimens, and recommended in every reference cited above, is to overlay a system of quality control or design rules for potential primer-probes sets. For example, the primer-probe sets have to yield a single band of the expected size as observed by gel electrophoresis and the sequence of the amplified product have to be identical to the expected product.

37. When these rules were applied with the design rules specified in the application and combined with the narrow requirements for amplification efficiency specified in the application, each primer-probe set met the requirements for the invention, i.e., each primer-probe set had high reproducibility (precision) and exhibited a calibrated or reproducible, ratiometric relationship to other assayed constituents in the selected panel, *in every tested case.*

38. As discussed above, even if a sequence at a different region of the same locus were chosen, the possible primer-probe combinations could be assessed with minimal experimentation and more than one primer-probe set could meet the conditions required for the invention; i.e. specificity and amplification efficiency.

39. Finally, regarding enablement in general, and the various reasons cited against this application for its lack thereof, it is my unqualified experience that those skilled in the art typically engage in the design and selection of primer-probes sets, they typically engage in experimental verification that the primer-probes sets so-designed and identified actually work as desired/required, and they typically engage in efforts to achieve the most efficient amplification technically feasible, which is determined experimentally. They also typically engage in screening very large numbers of genes from various organisms to develop gene expression profiles, as indicated by the enormous amount of literature on the subject. For example, when the term "gene expression profile" was plugged into the patent section of the USPTO web site by Applicants, as of May 5, 2003, such a search yielded 66 patents for the years 1976 to the present, and 305 patent applications for the years 2001 – 2003 alone!

CONSIDERATION OF THE PRIOR ART

Rodriquez-Antona et al 2000 does not anticipate or teach the invention

40. The cited reference (Rodriguez-Antona et al.), which deals with improving efficiency of reverse transcription in an RT-PCR system, is not particularly relevant to the discussion of amplification efficiency in this application. That is, reproducibility or efficiency of the reverse transcriptase step: (1) is not the same as amplification efficiency (as used in the application or the established art); (2) was well established in the art prior to submission of this application for uses similar to those in this application; and (3) is misapplied as germane to the question of anticipation of the presently claimed invention.

41. Further, the values depicted in Table III are not direct measurements but are rather ratios of molecules of mRNA/microgram of total RNA (see Rodriquez-Antona et al., p. 115, Table III). In turn, it appears that neither the numerator nor the denominator of this fraction is directly measured either, but rather both are the result of calculation and estimation. The numerator, number of molecules of mRNA, is determined by reference to a luciferase mRNA standard (see Rodriquez-Antona et al., p. 115, col. 1, 1st para.). The denominator appears to be derived from estimation as well.

42. Moreover, the variation listed in the individual derived values in Table III cannot be evaluated because no experimental details are provided. In particular, a calculated value of $(1100 \pm 100) \times 10^5$ for 2C9 does not tell us whether the error is the authors' estimate of accuracy of a single calculation or whether some number of determinations and calculations were made, and how variation of this sampling is reported. 38. There are more problems yet with Table III. We see results for four different liver samples for which no different physical or biological condition is identified, and the variation from

one sample to the next runs from about 300% to over 1000%. The large variability demonstrated in Table III of the reference is substantiated by the authors' own characterization of their results as being "in agreement with" results of previous experimenters who achieved results such as 3.4 ±2.4 for 1A2 (variation of 71%), 1.8 ± 1.9 for 2A6/7 (variation of 106%), etc.

43. Simple observation of the values in Table III shows variances of 45-fold (4500%) or greater for gene 2E1; 20-fold (2000%) or greater for gene 1A1; and 10-fold (1000%) or greater for genes 2C9, 3A4, and 3A5; etc. Further, Rodriguez-Antona et al claim that these results are similar to other published results for the same genes which show variations of 100% or more. Such extremely large fluctuations in interassay variability are totally unacceptable in the presently claimed system, which requires that the measurement of the constituents in the panel be obtained for each constituent under measurement conditions that are substantially reproducible. The inventors' believe that these unacceptable differences are likely due to differences of amplification efficiency among the samples assayed.

44. The content of other prior art references of record, designated as CV, CW, CX, CY and CZ in a supplemental Information Disclosure Statement filed December 23, 2003, indicates further the novel and non-obvious nature of the subject matter claimed herein. All these references, which reflect the state of the art, disclose a measurement technique in which the coefficients of variation are calculated only in relation to a ratio between an experimental target and a standard. No information is provided as to the actual coefficient of variation for repeated measurements of the same sample.

45. In fact, reference CZ regards its technique of providing an ability to detect with confidence changes of 20% in mRNA copy number as novel (see Zhang et al., p. 347, col. 1, first full para.). Moreover, this same reference teaches that restricting the range of amplification even within as much as 10% is undesirable because it results in exclusion of data (*id.*, p. 345, beginning of col. 2).

46. I hereby declare that all statements made herein are of my own knowledge and that all statements made on information and belief are true; and further that these statements are being made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Victor V. Tryon Ph.D.

Victor V. Tryon, Ph.D.

Dated: May 27, 2003 VT

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